

ins.D)
ins.
G

PROCESS FOR THE HETEROTROPHIC PRODUCTION OF
MICROBIAL PRODUCTS WITH HIGH CONCENTRATIONS OF
OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS

5 Cross-Reference to Related Applications

10 This application is a continuation-in-part of copending
and commonly assigned U.S. patent application Serial No.
07/911,760, filed July 10, 1992 which is incorporated herein
in its entirety by reference which is a divisional of U.S.
patent application Serial No. 07/580,778, filed September
11, 1990 which is incorporated herein in its entirety which
issued as U.S. Patent No. 5,130,242 which is a continuation-
in-part of copending and commonly assigned U.S. patent
application Serial No. 07/439,093, filed November 17, 1989
15 which is incorporated herein in its entirety by reference
which is a continuation-in-part of U.S. Patent Application
Serial No. 07/241,410, filed September 7, 1988 which was
previously expressly abandoned.

20 Field of the Invention

The field of this invention relates to heterotrophic
organisms and a process for culturing them for the production
of lipids with high concentrations of omega-3 highly
unsaturated fatty acids (HUFA) suitable for human and animal
25 consumption as food additives or for use in pharmaceutical
and industrial products.

Background of the Invention

Omega-3 highly unsaturated fatty acids (HUFAs) are of
30 significant commercial interest in that they have been

recently recognized as important dietary compounds for preventing arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells. These beneficial effects are a result both of omega-3 HUFAS causing competitive inhibition of compounds produced from omega-6 fatty acids, and from beneficial compounds produced directly from the omega-3 HUFAS themselves (Simopoulos et al., 1986). Omega-6 fatty acids are the predominant HUFAS found in plants and animals. Currently, a commercially available dietary source of omega-3 HUFAS is from certain fish oils which can contain up to 20-30% of these fatty acids. The beneficial effects of these fatty acids can be obtained by eating fish several times a week or by daily intake of concentrated fish oil. Consequently large quantities of fish oil are processed and encapsulated each year for sale as a dietary supplement. However, there are several significant problems with these fish oil supplements, including bioaccumulation of fat-soluble vitamins and high levels of saturated and omega-6 fatty acids, both of which can have deleterious health effects.

Another source of omega-3 HUFAS is the microflora *Thraustochytrium* and *Schizochytrium* which are discussed in detail in related U.S. Patent No. 5,130,242. These microflora have the advantages of being heterotrophic and capable of high levels of omega-3 HUFA production. There still exists a need however for improved methods for

fermentation of these microflora and identification of improved uses of the microflora product.

Brief Summary of the Invention

5 The present invention is directed to a new process for growing the microflora *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, which includes the growing of the microflora in a culture medium containing non-chloride containing sodium salts, particularly including sodium sulfate. More particularly, a significant portion of the sodium requirements of the fermentation are supplied as a non-chloride containing sodium salt. The present process is particularly useful in commercial production because the chloride content in the medium can be significantly reduced, thereby avoiding the corrosive effects of chloride on fermentation equipment. In addition, the present invention is particularly useful for production of food products for use in aquaculture because *Thraustochytrium* and *Schizochytrium* cultured in such media form much smaller clumps than those cultured in high chloride media and are thus more available as a food source for larval shrimp. In particular, *Thraustochytrium* and *Schizochytrium* cultured in medium containing sodium sulfate can have cell aggregates of an average size of less than about 150 microns in diameter.

A further embodiment of the present invention is the production of a microflora biomass comprising *Thraustochytrium*, *Schizochytrium*, and mixtures thereof which

have an average cell aggregate size of less than about 150 microns. The microflora biomass is useful for aquaculture and in particular, for feeding larval shrimp because the microflora have the primary feed advantages required for
5 shrimp of a high sterol content and a high omega-3 highly unsaturated fatty acid (HUFA) content. Additionally, because of the small cell aggregate size, the microflora can be eaten by the larval shrimp, brine shrimp, rotifers, and mollusks. The present invention further includes a process for the
10 production of these organisms which includes feeding *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, having an average cell size of less than about 150 microns to them.

A further embodiment of the present invention is
15 directed to a food product which is comprised of microflora selected from the group consisting of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof and an additional component selected from the group consisting of flaxseed, rapeseed, soybean, avocado meal, and mixtures thereof. A
20 particular advantage of this food product is that it has a high long chain omega-3 fatty acid content and a high short chain omega-3 fatty chain content from the additional component. In a further embodiment, the food product is produced by extrusion. The extrusion process involves mixing
25 the microflora with the additional component, thereby reducing the moisture content of the food product. The food product is then extruded under heat, thus driving off a significant portion of the reduced moisture. The remaining

amount of the original moisture content is readily removed by air drying or short baking times, thereby reducing the overall energy requirements of drying and the potential degradation of the omega-3 HUFA's by extended drying at high
5 temperatures.

Brief Description of the Figures

Fig. 1 is a graphical representation of HUFA production in newly isolated strains of the invention, represented by
10 ■, and previously isolated strains represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 HUFAs (abscissa) and the percent by weight of total fatty acids which were omega-6 fatty acids
15 (ordinate). Only those strains of the invention were plotted wherein less than 10.6% (w/w) of total fatty acids were omega-6 and more than 67% of total fatty acids were omega-3.

Fig. 2 is a graphical representation of HUFA production in newly isolated strains of the invention, represented by
20 ■, and previously isolated strains, represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 HUFAs (abscissa) and percent of weight of total fatty acids which were eicosapentaenoic acid (EPA
25 C20:5n-3) (ordinate). Only those strains of the invention were plotted wherein more than 67% (w/w) of total fatty acids were omega-3 and more than 7.8% (w/w) of total fatty acids were C20:5n-3.

Fig. 3 is a graphical representation of omega-3 HUFA composition in newly isolated strains of the invention, represented by \square , and previously isolated strains, represented by +. Each point represents a separate strain.

5 Values on the abscissa are weight fraction of total omega-3 HUFAs which were C20:5n-3 and on the ordinate are weight fraction of total omega-3 fatty highly unsaturated acids which were C22:6n-3. Only strains of the invention were plotted having either a weight fraction of C20:5n-3 28% or
10 greater, or a weight fraction of C22:6n-3 greater than 93.6%.

Fig. 4 is a graph showing growth of various newly isolated strains of the invention and previously isolated strains, at 25°C and at 30°C. Growth rates are normalized to the growth rate of strain U-30 at 25°C. Previously
15 isolated strains are designated by their ATCC accession numbers.

Fig. 5 is a graph of total yields of cellular production after induction by nitrogen limitation. Each of ash-free dry weight, total fatty acids and omega-3 HUFAs, as
20 indicated, was plotted, normalized to the corresponding value for strain 28211. All strains are identified by ATCC accession numbers.

Fig. 6 is a graph of fatty acid yields after growth in culture media having the salinity indicated on the abscissa.
25 Strains shown are newly isolated strains S31 (ATCC 20888) (\square) and U42-2 (ATCC 20891) (+) and previously isolated strains, ATCC 28211 (\diamond) and ATCC 28209 (Δ). Fatty acid yields are plotted as relative yields normalized to an

arbitrary value of 1.00 based on the average growth rate exhibited by S31 (ATCC 20888) (\square) over the tested salinity range.

Fig. 7 is a graph of increases in the omega-3 HUFA content of the total lipids in the brine shrimp, *Artemia salina*, fed Thraustochytrid strain (ATCC 20890) isolated by the method in Example 1. EPA = C20:5n-3; DHA = C22:5n-3.

Fig. 8 is a graph of increases in the omega-3 HUFA content of the total lipids in the brine shrimp, *Artemia salina*, fed Thraustochytrid strain (ATCC 20888) isolated by the method in Example 1. EPA = C20:5n-3; DHA = C22:5n-3.

Detailed Description of the Preferred Embodiments

For purposes of definition throughout the application, it is understood herein that a fatty acid is an aliphatic monocarboxylic acid. Lipids are understood to be fats or oils including the glyceride esters of fatty acids along with associated phosphatides, sterols, alcohols, hydrocarbons, ketones, and related compounds.

A commonly employed shorthand system is used in this specification to denote the structure of the fatty acids (e.g., Weete, 1980). This system uses the letter "C" accompanied by a number denoting the number of carbons in the hydrocarbon chain, followed by a colon and a number indicating the number of double bonds, i.e., C20:5, eicosapentaenoic acid. Fatty acids are numbered starting at the carboxy carbon. Position of the double bonds is indicated by adding the Greek letter delta (Δ) followed by

the carbon number of the double bond; i.e., C20:5 ω -3 $\Delta^{5,8,11,14,17}$. The "omega" notation is a shorthand system for unsaturated fatty acids whereby numbering from the carboxy-terminal carbon is used. For convenience, n-3 will be used to symbolize "omega-3," especially when using the numerical shorthand nomenclature described herein. Omega-3 highly unsaturated fatty acids are understood to be polyethylenic fatty acids in which the ultimate ethylenic bond is 3 carbons from and including the terminal methyl group of the fatty acid. Thus, the complete nomenclature for eicosapentaenoic acid, an omega-3 highly unsaturated fatty acid, would be C20:5n-3 $\Delta^{5,8,11,14,17}$. For the sake of brevity, the double bond locations ($\Delta^{5,8,11,14,17}$) will be omitted. Eicosapentaenoic acid is then designated C20:5n-3, Docosapentaenoic acid (C22:5n-3 $\Delta^{7,10,13,16,19}$) is C22:5n-3, and Docosahexaenoic acid (C22:6n-3 $\Delta^{4,7,10,13,16,19}$) is C22:6n-3. The nomenclature "highly unsaturated fatty acid" means a fatty acid with 4 or more double bonds. "Saturated fatty acid" means a fatty acid with 1 to 3 double bonds.

A collection and screening process has been developed to readily isolate many strains of microorganisms with the following combination of economically desirable characteristics for the production of omega-3 HUFAs: 1) capable of heterotrophic growth; 2) high content of omega-3 HUFAs; 3) unicellular; 4) preferably low content of saturated and omega-6 HUFAs; 5) preferably nonpigmented, white or essentially colorless cells; 6) preferably thermotolerant (ability to grow at temperatures above 30°C); and 7)

preferably euryhaline (able to grow over a wide range of salinities, but especially at low salinities). This process is described in detail in related U.S. Patent No. 5,130,242.

enc B
5 Using the collection and screening process, strains of unicellular microflora can be isolated which have fatty acid contents up to about 45% total cellular dry weight percent (%dwt), and which exhibit growth over a temperature range from 15-48°C and grow in a very low salinity culture medium. Many of the very high omega-3 strains are very slow growers.
10 Strains which have been isolated by the method outlined above, and which exhibit rapid growth, good production and high omega-3 HUFA content, have omega-3 unsaturated fatty acid contents up to approximately 12% dwt.

One aspect of the present invention is the growth of
15 *Thraustochytrium*, *Schizochytrium*, and mixtures thereof with high omega-3 HUFA content, in fermentation medium containing non-chloride containing sodium salts and preferably sodium sulfate. More particularly, a significant portion of the sodium requirements of the fermentation are supplied as non-
20 chloride containing sodium salts. For example, less than about 75% of the sodium in the fermentation medium is supplied as sodium chloride, more preferably less than about 50% and more preferably less than about 25%. A particular advantage of the present invention is that the medium
25 provides the source of sodium needed by the microflora to grow in the absence of a significant amount of chloride which can corrode the vessel in which the microflora are being grown and other fermentation or downstream processing

equipment. It has been surprisingly found that microflora of the present invention can be grown at chloride concentrations of less than about 3 g/l, more preferably less than about 500 mg/l, more preferably less than about 250 mg/l and more preferably between about 60 mg/l and about 120 mg/l while still attaining high yields of biomass per sugar of about 50% or greater. As discussed below, an additional advantage of the present invention is the production of microflora that are high in omega-3 HUFA content but have a small enough cell aggregate size to be consumed by larval shrimp, brine shrimp, rotifers and mollusks.

Non-chloride containing sodium salts can include soda ash (a mixture of sodium carbonate and sodium oxide), sodium carbonate, sodium bicarbonate, sodium sulfate and mixtures thereof, and preferably include sodium sulfate. Soda ash, sodium carbonate and sodium bicarbonate tend to increase the pH of the fermentation medium, thus requiring control steps to maintain the proper pH of the medium. The concentration of sodium sulfate is effective to meet the salinity requirements of the microflora, preferably the sodium concentration is (expressed as g/l of Na) is greater than about 1.0 g/l, more preferably between about 1.0 g/l and about 50.0 g/l and more preferably between about 2.0 g/l and about 25 g/l.

It has been surprisingly found that fermentation of the strains in the presence of a non-chloride containing sodium salt and particularly, sodium sulfate limits the cell

aggregate size of the strains to less than about 150 microns, preferably less than about 100 microns, and more preferably less than about 50 microns. As used herein, the term cell aggregate size refers to the approximate average diameter of clumps or aggregates of cells in a fermentation medium of a microfloral culture. Typically, greater than about 25 percent of the cell aggregates in a microfloral culture have cell aggregate size below the average size, more preferably greater than about 50 percent and more preferably greater than about 75 percent. Microfloral cells produced in accordance with the present invention meet cell aggregate size parameters described above while in fermentation medium as well as after freezing and/or drying of the biomass if resuspended in liquid or physically agitated, such as by a blender or vortexer. The present process is particularly important for microflora which replicate by successive bipartition (wherein a single cell replicates by dividing into two cells which each divide into two more, etc.) because as cells repeatedly and rapidly undergo this process, the cells tend to clump forming multi-cell aggregates which are often outside the cell aggregate size parameters identified above. *Schizochytrium* replicate by successive bipartition and by forming sporangia which release zoospores. *Thraustochytrium*, however, replicate only by forming sporangia and releasing zoospores. For *Thraustochytrium* which replicate by sporangia/zoospore formation, clumping can be a problem as well, particularly because even though the number of cells in an aggregate may not be as great as

aggregates formed by successive bipartition, the individual cell sizes of *Thraustochytrium* tend to be larger, and thus, clumps of a small number of cells are larger. However, one deposited strain of *Thraustochytrium*, ATCC 26185, has been
5 identified which does not exhibit significant aggregation.

In another aspect of the present invention, it has been found that by restricting the oxygen content of the fermentation medium during the growth of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, the lipid content of
10 the strains can be increased. The optimum oxygen concentration for lipid production can be determined for any particular microflora by variation of the oxygen content of the medium. In particular, the oxygen content of the fermentation medium is maintained at an oxygen content of
15 less than about 40% of saturation and preferably between about 5% of saturation and about 40% of saturation.

Growth of the strains by the invention process can be effected at any temperature conducive to satisfactory growth of the strains; for example, between about 5°C and about
20 48°C, preferably between about 15°C and about 40°C, and more preferably between about 25°C and about 35°C. The culture medium typically becomes more alkaline during the fermentation if pH is not controlled by acid addition or buffers. The strains will grow over a pH range from 5.0-11.0
25 with a preferable range of about 6.0-8.5.

Various fermentation parameters for inoculating, growing and recovering microflora are discussed in detail in U.S. Patent No. 5,130,242. The biomass harvested from a

fermentation run can be dried (e.g., spray drying, tunnel drying, vacuum drying, or a similar process) and used as a feed or food supplement for any animal whose meat or products are consumed by humans. Similarly, extracted omega-3 HUFAs can be used as a feed or food supplement. Alternatively, the harvested and washed biomass can be used directly (without drying) as a feed supplement. To extend its shelf life, the wet biomass can be acidified (approximate pH = 3.5-4.5) and/or pasteurized or flash heated to inactivate enzymes and then canned, bottled or packaged under a vacuum or non-oxidizing atmosphere (e.g., N₂ or CO₂). The term "animal" means any organism belonging to the kingdom Animalia and includes, without limitation, any animal from which poultry meat, seafood, beef, pork or lamb is derived. Seafood is derived from, without limitation, fish, shrimp and shellfish. The term "products" includes any product other than meat derived from such animals, including, without limitation, eggs or other products. When fed to such animals, omega-3 HUFAs in the harvested biomass or extracted omega-3 HUFAs are incorporated into the flesh, eggs or other products of such animals to increase the omega-3 HUFA content thereof.

A further embodiment of the present invention is the use of the harvested biomass as a food product for larval shrimp, brine shrimp, rotifers and mollusks and in particular, larval shrimp. During the larval stage of development, shrimp larvae are unable to use some food sources because the food source is too large. In particular, at certain stages of development, shrimp larvae are unable

to use a food source having a diameter greater than about 150 microns. Thus, microflora grown in fermentation medium containing a non-chloride sodium salt, and particularly sodium sulfate, as broadly discussed above, are suitable for use as a shrimp food product. As discussed above, microflora grown under such conditions typically have a cell aggregate size less than about 150 microns, preferably less than about 100 microns, and more preferably less than about 50 microns.

A further advantage of the use of microflora of the present invention as a food source for shrimp is that such microflora have a significant sterol content including cholesterol, which is a primary feed requirement for shrimp. The microflora of the present invention typically have a sterol content of preferably at least about 0.1% ash-free dry weight (afdwt), more preferably at least about 0.5% afdwt, and even more preferably at least about 1.0% afdwt. In addition, the microflora of the present invention typically have a cholesterol content of preferably at least about 15% of the total sterol content, more preferably at least about 25% of the total sterol content, and even more preferably at least about 40% of the total sterol content. Further, the microfloral biomass of the present invention also provide shrimp with additional nutritional requirements such as omega-6 fatty acids, protein, carbohydrates, pigments and vitamins.

The microbial product of the present invention is of value as a source of omega-3 HUFAs for fish, shrimp and other products produced by aquaculture. The product can be used

as a food product as described above for shrimp; or added directly as a supplement to the feed for shrimp and fish, generally; or it can be fed to brine shrimp or other live feed organisms intended for consumption by an aquacultured organism. The use of such microflora in this manner enables the shrimp farmer to obtain significantly higher growth rates and/or survival rates for larval shrimp and to produce post-larval shrimp which are more hardy and robust.

For most feed applications, the fatty acid content of the harvested cells will be approximately 15-50% dwt with the remaining material being largely protein and carbohydrate. The protein can contribute significantly to the nutritional value of the cells as several of the strains that have been evaluated have all of the essential amino acids and would be considered a nutritionally balanced protein.

A further embodiment of the present invention is the production of a food product using the *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, of the present invention, combined with an additional component selected from the group consisting of rapeseed, flaxseed, soybean and avocado meal. A particular advantage of this embodiment is that the food product contains both short chain omega-3 HUFAS from the additional component and long chain omega-3 HUFAS from the microflora. Food products having flaxseed, rapeseed, soybeans and avocado meal are known to be useful for supplying a source of short chain omega-3 HUFAS and for additionally supplying a source of short chain omega-3 HUFAS,

which can be elongated by the humans and animals that ingest them. Such food products, however, have the disadvantages of having high choline contents from the additional component, which can form primary amines and result in an unpleasant fish smell; and toxic compounds from the additional component, which at high levels can, for example, inhibit the laying of eggs by hens or cause animals to go off of their feed. As such, the food product of the present invention has the advantage of a lowered flaxseed, rapeseed, soy bean or avocado meal content because the organism ingesting the food product does not need high levels of short chain omega-3 HUFAs for the purpose of converting them to long chain HUFAs. Thus, the lowered content of the flaxseed and rapeseed of the food product results in lowered amounts of choline and/or inhibitory toxic compounds present in the food product.

The amount of *Thraustochytrium*, *Schizochytrium*, and mixtures thereof, used in the food product can range from between about 5% to about 95% by weight. The additional component can be present in the food product at a range of between about 5% to about 95% by weight. Additionally, the food product can include other components as well, including grains, supplements, vitamins, binders and preservatives.

In a preferred embodiment, the above food product is produced using an extrusion process. The extrusion process involves mixing the microflora with the additional component, thereby reducing the moisture in the microfloral biomass by the amount of the additional component mixed. The food

product is extruded under heat, thus removing further moisture from the food product. The resulting product which has a low moisture content can be air dried or dried by relatively short baking times thereby reducing the overall energy requirements of drying and the potential degradation of omega-3 HUFAS due to long time periods at high temperatures. In addition, heat from the extrusion process can degrade some of the unwanted toxic compounds commonly found in the additional component which can, for example, inhibit egg laying by hens or cause animals to go off of their feed.

The present invention will be described in more detail by way of working examples. Species meeting the selection criteria described above have not been described in the prior art. By employing these selection criteria, over 25 potentially promising strains have been isolated from approximately 1000 samples screened. Out of the approximate 20,500 strains in the American Type Culture Collection (ATCC), 10 strains were later identified as belonging to the same taxonomic group as the strains isolated. Those strains still viable in the Collection were procured and used to compare with strains isolated and cultured by the disclosed procedures. The results of this comparison are presented in Examples 4 and 5 below.

The most recent taxonomic theorists place Thraustochytrids with the algae or algae-like protists. All of the strains of unicellular microorganisms disclosed and claimed herein are members of the order Thraustochytriales

(Order: Thraustochytriales; Family: Thraustochytriaceae; Genus: *Thraustochytrium* or *Schizochytrium*). For general purposes of discussion herein, these microorganisms will be called microflora to better denote their uncertain exact taxonomic position.

The novel strains identified below were deposited under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. All restrictions on the availability to the public of the materials so deposited will be irrevocably removed upon the granting of a patent. Each deposit will be stored for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism is received by the American Type Culture Collection (ATCC), and, in any case, for a period of at least 30 years after the date of the deposit.

Preferred microorganisms of the present invention have all of the identifying characteristics of the deposited strains and, in particular, the identifying characteristics of being able to produce omega-3 HUFAs as described herein and having cell aggregate size characteristics when cultured under conditions as described herein. In particular, the preferred microorganisms of the present invention refer to the following deposited microorganisms and mutants thereof.

<u>Strain</u>	<u>ATCC No.</u>	<u>Deposit Date</u>
<i>Schizochytrium</i> S31	20888	8/8/88
<i>Schizochytrium</i> S8	20889	8/8/88

30

EXAMPLES

Example 1. Collection and Screening

A 150ml water sample was collected from a shallow, inland saline pond and stored in a sterile polyethylene bottle. Special effort was made to include some of the living plant material and naturally occurring detritus (decaying plant and animal matter) along with the water sample. The sample was placed on ice until return to the laboratory. In the lab, the water sample was shaken for 15-30 seconds, and 1-10ml of the sample was pipetted or poured into a filter unit containing 2 types of filters: 1) on top, a sterile 47mm diameter Whatman #4 filter having a pore size about $25\mu\text{m}$; and 2) underneath the Whatman filter, a 47mm diameter polycarbonate filter with about $1.0\mu\text{m}$ pore size. Given slight variations of nominal pore sizes for the filters, the cells collected on the polycarbonate filter range in size from about $1.0\mu\text{m}$ to about $25\mu\text{m}$.

The Whatman filter was removed and discarded. The polycarbonate filter was placed on solid F-1 media in a petri plate, said media consisting of (per liter): 600ml seawater (artificial seawater can be used), 400ml distilled water, 10g agar, 1g glucose, 1g protein hydrolysate, 0.2g yeast extract, 2ml 0.1 M KH_2PO_4 , 1ml of a vitamin solution (A-vits) (Containing 100mg/l thiamine, 0.5mg/l biotin, and 0.5mg/l cyanocobalamin), 5ml of a trace metal mixture (PII metals, containing per liter: 6.0g Na_2EDTA , 0.29g $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, 6.84g H_3BO_3 , 0.86 $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 0.06g ZnCl_2 , 0.026g $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, (0.052g

applications. Strains with high omega-6 content can also be isolated. Strains of these microflora can be repeatedly isolated from the same location using the procedure outlined above. A few of the newly isolated strains have very similar fatty acid profiles. The possibility that some are duplicate isolates of the same strain cannot be ruled out at present. Further screening for other desirable traits such as salinity tolerance or ability to use a variety of carbon and nitrogen sources can then be carried out using a similar process.

10

Example 2. Maintaining Unrestricted Growth: PO₄ and Yeast Extract

Cells of *Schizochytrium aggregatum* (ATCC 28209) were picked from solid F-1 medium and inoculated into 50ml of FFM medium. (Fuller et al., 1964). This medium contains: seawater, 1000ml; glucose, 1.0g; gelatin hydrolysate, 1.0g; liver extract, 0.01g; yeast extract, 0.1g; PII metals, 5ml; 1ml B-vitamins solution (Goldstein et al., 1969); and 1ml of an antibiotic solution (25g/l streptomycin sulfate and penicillin-G). 1.0ml of the vitamin mix (pH 7.2) contains: thiamine HCl, 200µg; biotin, 0.5µg; cyanocobalamin, 0.05µg; nicotinic acid, 100µg; calcium pantothenate, 100µg; riboflavin, 5.0µg; pyridoxine HCl, 40.0µg; pyridoxamine 2HCl, 20.0µg; p-aminobenzoic acid, 10µg; chlorine HCl, 500µg; inositol, 1.0mg; thymine, 0.8mg; orotic acid, 0.26mg; folinic acid, 0.2µg; and folic acid, 2.5µg. The culture was placed on a rotary shaker (200 rpm) at 27°C. After 3-4 days, 1ml of this culture was transferred to 50ml of each of the following treatments: 1) FFM medium (as control); and 2) FFM

medium with the addition of 250mg/l KH_2PO_4 and 250mg/l yeast extract. These cultures were placed on a rotary shaker (200 rpm) at 27°C for 48 hr. The cells were harvested and the yield of cells quantified. In treatment 1, the final concentration of cells on an ash-free dry weight basis was 616mg/l. In treatment 2, the final concentration of cells was 1675mg/l, demonstrating the enhanced effect of increasing PO_4 and yeast extract concentrations in the culture medium.

10 Example 3. Maintaining Unrestricted Growth: Substitution of Corn Steep Liquor for Yeast Extract

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888) were picked from solid F-1 medium and placed into 50ml of M-5 medium. This medium consists of (on a per liter basis):
15 yeast extract, 1g; NaCl, 25g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5g; KCl, 1g; CaCl_2 , 200mg; glucose, 5g; glutamate, 5g; KH_2PO_4 , 1g; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotic solution, 1ml. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. Sterile solutions of corn steep liquor (4g/40ml; pH 7.0) and yeast extract (1g/40ml; pH 7.0)
20 were prepared. To one set of M-5 medium flasks, the following amount of yeast extract solution was added: 1) 2ml; 2) 1.5ml; 3) 1ml; 4) 0.5ml; and 5) 0.25ml. To another set of M-5 medium flasks the yeast extract and corn steep liquor solutions were added at the following levels: 1) 2ml yeast
25 extract; 2) 1.5ml yeast extract and 0.5ml corn steep liquor; 3) 1.0ml yeast extract and 1.0ml corn steep liquor; 4) 0.5ml yeast extract and 1.5ml corn steep liquor; and 5) 2ml corn steep liquor. One ml of the culture in F-1 medium was used

to inoculate each flask. They were placed on a rotary shaker at 27°C for 48 hr. The cells were harvested by centrifugation and the yield of cells (as ash-free dry weight) was determined. The results are shown in Table 1.

5 The results indicate the addition of yeast extract up to 0.8g/l of medium can increase the yield of cells. However, addition of corn steep liquor is even more effective and results in twice the yield of treatments with added yeast extract. This is very advantageous for the economic
10 production of cells as corn steep liquor is much less expensive than yeast extract.

Table 1.

15	Treatment (Amount Nutrient Supplement Added)	Ash-Free Dry Weight (mg/l)
20	2.0ml yeast ext.	4000
	1.5ml yeast ext.	4420
	1.0ml yeast ext.	4300
25	0.5ml yeast ext.	2780
	0.25ml yeast ext.	2700
30	2.0ml yeast ext.	4420
	1.5ml yeast ext. + 0.5ml CSL*	6560
35	1.0ml yeast ext. + 1.0ml CSL	6640
	0.5ml yeast ext. + 1.5ml CSL	7200
40	2.0ml CSL	7590

*CSL = corn steep liquor

Example 4. Enhanced HUFA Content of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Previously Known Strains)

5 A battery of 151 newly isolated strains, selected according to the method described in Example 1, were sampled in late exponential phase growth and quantitatively analyzed for HUFA content by gas-liquid chromatography. All strains were grown either in M1 medium or liquid FFM medium, whichever gave highest yield of cells. M1 medium has the
10 same composition as M5 medium, except that the concentrations of glucose and glutamate are 1 g/l. Additionally, five previously isolated *Thraustochytrium* or *Schizochytrium* species were obtained from the American Type Culture Collection, representing all the strains which could be
15 obtained in viable form from the collection. These strains were: *T. aureum* (ATCC No. 28211), *T. aureum* (ATCC No. 34304), *T. roseum* (ATCC No. 28210), *T. straitum* (ATCC No. 34473) and *S. aggregatum* (ATCC No. 28209). The strains all exhibited abbreviated growth in conventional media, and generally
20 showed improved growth in media of the present invention, including M5 medium and FFM medium. The fatty acid production of each of the known strains was measured as described, based upon the improved growth of the strains in media of the invention.

25 Fatty acid peaks were identified by the use of pure compounds of known structure. Quantitation, in terms of percent by weight of total fatty acids, was carried out by integrating the chromatographic peaks. Compounds identified were: palmitic acid (C16:0), C20:4n-6 and C22:1 (which were

not resolved separately by the system employed), C20:5n-3, C22:5n-6, C22:5n-3, and C22:6n-3. The remainder, usually lower molecular weight fatty acids, were included in the combined category of "other fatty acids." Total omega-3 fatty acids were calculated as the sum of 20:5n-3, 22:5n-3 and 22:6n-3. Total omega-6 fatty acids were calculated as the sum of the 20:4/22:1 peak and the 22:5n-6 peak.

The results are shown in Tables 2-3 and illustrated in Figs. 1-3. From Table 2 it can be seen that large numbers of strains can be isolated by the method of the invention, and that large numbers of strains outperform the previously known strains by several important criteria. For example, 102 strains produced at least 7.8% by weight of total fatty acids C20:5w3, a higher percentage of that fatty acid than any previously known strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. Thirty (30) strains of the invention produced at least 68% by weight of total fatty acids as omega-3 fatty acids, more than any previously known strain. Strain 23B (ATCC No. 20892) is an example of such strains. Seventy-six (76) strains of the invention yielded not more than 10% by weight of total fatty acids as omega-6 fatty acids, considered undesirable components of the human diet, lower than any previously known strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. In addition, there are 35 strains of the invention that produce more than 25% by weight of total fatty acids as omega-6 fatty acids, more than any previously known strain. While such

strains may have a more narrow range of uses for dietary purposes, they are useful as feedstock for chemical synthesis of eicosanoids starting from omega-6 fatty acids.

In addition, the data reveal many strains of the
5 invention which produce a high proportion of total omega-3 fatty acids as C22:6n-3. In Table 3, 48 of the strains shown in Table 2 were compared to the previously known strains, showing each of C20:5n-3, C22:5n-3 and C22:6n-3 as percent by weight of total omega-3 content. Fifteen strains had at
10 least 94% by weight of total omega-3 fatty acids as C22:6n-3, more than any previously known strain. Strain S8 (ATCC No. 20889) was an example of such strains. Eighteen strains had at least 28% by weight of total omega-3 fatty acids as C20:5n-3, more than any previously known strain. Strain 12B
15 (ATCC No. 20890) was an example of such strains.

TABLE 2: LIST OF STRAINS AND COMPOSITIONS UNDER STANDARD SCREENING CONDITIONS

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.41	2.01	6.61	3.21	0.21	8.31	48.51	15.11	6.01	21
22.91	0.41	2.31	15.51	0.51	47.01	11.51	49.71	15.91	AICC20089
14.91	6.51	12.01	11.81	0.41	49.71	4.71	62.11	18.31	U40-2
40.31	1.71	3.81	8.61	0.01	8.71	37.41	12.01	10.21	210
20.71	0.41	7.01	0.01	0.01	1.11	70.11	0.21	0.41	BGI
26.01	5.71	1.51	9.71	0.71	9.71	46.71	11.21	15.41	56A
16.41	1.41	10.01	1.91	2.21	46.41	21.01	58.61	3.31	11A-1
23.71	3.31	10.51	1.91	1.81	29.91	20.91	42.21	5.21	4A-1
18.71	6.91	9.21	11.91	3.21	25.21	24.91	37.51	18.81	170
15.41	4.21	7.31	9.51	0.91	51.21	11.61	59.31	13.71	AICC20091
22.31	3.91	7.61	23.51	0.51	22.11	20.21	30.21	27.41	544
14.41	2.31	15.01	18.41	0.71	43.01	5.51	59.41	20.71	U10
22.11	7.01	3.11	12.71	1.01	14.91	38.31	19.01	20.51	59A
18.11	2.31	6.91	9.11	0.01	52.21	10.61	59.91	11.41	U37-2
15.81	3.91	8.81	11.61	1.21	53.31	5.51	63.31	15.51	550W
23.71	3.81	6.31	6.91	0.61	43.01	15.61	50.01	10.71	AICC20091
18.01	0.01	0.01	0.01	0.01	0.01	90.01	0.01	0.01	UX
16.61	6.31	11.91	13.31	1.71	43.01	7.31	56.61	19.51	UW9
17.31	2.31	8.41	11.41	0.71	53.61	6.51	62.61	13.61	C32-2
23.01	1.21	6.41	2.51	1.91	34.41	29.81	42.61	3.71	5A-1
17.11	5.21	11.11	7.61	2.21	27.21	29.61	40.41	12.91	BGI
25.41	2.21	9.61	7.01	1.11	46.01	8.01	56.71	9.11	U3
16.91	12.01	6.61	16.21	0.41	25.11	22.81	32.11	20.21	550
26.31	2.61	8.61	2.01	2.51	32.41	25.51	43.51	4.61	10A
19.41	0.31	9.81	0.01	0.31	38.41	31.71	48.61	0.31	320
16.01	16.71	0.61	18.41	0.01	22.51	17.71	31.11	35.11	560
18.61	7.71	11.41	3.61	4.31	31.71	22.71	47.41	11.21	5X2
17.01	4.41	16.21	6.41	3.71	33.61	17.81	53.51	10.91	530
16.81	2.71	13.81	20.51	1.41	32.31	5.51	54.41	23.31	549
20.81	8.01	8.91	6.41	1.71	33.91	20.31	44.51	14.41	53
14.81	0.31	3.71	3.91	0.01	69.91	7.41	73.61	4.21	3A-1
28.11	5.21	12.71	3.21	0.91	20.91	29.01	34.51	8.41	15A
20.91	0.71	8.51	1.01	0.01	35.01	39.01	44.31	1.71	9A-1
15.71	10.21	8.81	13.41	1.51	23.91	26.31	34.31	23.71	510
16.21	11.21	7.81	16.41	1.51	20.41	26.51	29.71	27.61	8A-1
20.51	5.51	8.61	4.81	2.71	28.71	29.21	40.01	10.31	13A-1
16.11	13.61	11.11	16.01	0.01	28.41	14.81	39.41	29.61	240-2
16.91	7.31	16.41	6.11	0.01	40.81	12.41	57.21	13.41	240-1
16.21	0.01	10.91	1.01	0.01	56.51	15.51	67.41	1.01	30
17.01	0.01	5.01	2.31	0.01	73.41	2.31	78.31	2.31	50G5
20.81	4.51	5.81	3.81	1.01	22.71	41.31	29.51	8.41	160
19.01	14.01	8.31	18.91	0.71	23.91	15.21	32.71	32.91	6A-1
18.01	0.31	10.11	0.01	0.01	48.91	22.71	59.01	0.31	330

The figure consists of 12 sub-diagrams labeled (a) through (l), arranged vertically. Each diagram shows a different stage in the construction of a 3D model of a human head and neck. The models are rendered in a wireframe style with some shading to indicate depth and form. The sequence starts with a basic head shape and gradually adds more detail, including facial features like eyes, nose, and mouth, as well as the neck and jawline. The final model (l) appears to be a more complete representation of a human head and neck.

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C18:0	C18:1	C22:0	C22:1	C22:6	Other FA	Omega3	Omega6	
16.71	5.51	14.01	0.51	1.71	31.01	21.01	40.31	13.91	840
15.01	1.01	11.71	2.11	0.91	62.31	6.91	74.91	3.11	28A
17.01	10.51	0.11	20.51	0.01	22.11	12.91	30.21	39.01	430
16.91	0.01	3.41	2.71	0.01	61.21	15.01	64.61	2.71	1A-1
15.61	2.71	11.41	10.91	0.01	53.71	4.91	65.91	13.61	U41-2
16.51	0.71	3.91	3.91	0.01	60.41	6.71	72.21	4.61	560
14.41	0.91	10.91	2.51	1.01	66.41	3.01	70.31	3.41	46A
17.61	0.01	2.41	3.31	0.01	66.31	10.41	60.71	3.31	15A-1
25.01	0.01	3.31	0.01	1.41	53.21	17.11	57.91	0.01	13A
16.11	13.41	9.31	13.61	0.01	32.31	15.31	41.61	27.01	370
16.51	9.11	13.21	6.71	0.01	30.91	15.61	52.11	15.91	430
16.11	12.41	12.01	15.71	0.01	30.51	12.51	43.31	20.11	170
13.01	0.01	11.51	2.01	0.01	67.01	4.11	70.61	3.61	27A
17.51	10.61	9.01	19.51	0.01	21.71	13.71	30.71	30.11	460
21.41	1.41	10.91	0.01	5.01	43.51	9.91	67.31	1.41	ATCC20020
17.71	0.01	0.61	4.41	0.01	60.21	9.11	60.01	4.41	5A
17.61	16.01	9.61	10.01	0.01	25.61	12.41	35.21	34.01	700-2
14.01	0.91	13.21	1.61	0.01	64.71	5.51	77.91	2.61	270
19.51	2.91	16.61	1.11	1.61	30.21	20.11	40.51	4.01	490
17.21	0.71	6.01	2.71	0.01	63.01	9.61	69.01	3.41	100
14.41	3.51	13.51	26.01	1.01	37.21	4.41	51.61	29.51	549-2
16.11	2.21	15.71	21.61	0.01	36.71	7.01	52.41	23.71	200
17.31	4.71	14.31	7.21	2.91	30.21	23.51	47.31	11.91	00
11.51	3.31	11.31	6.51	1.11	59.91	6.51	72.21	9.01	130
16.61	0.71	10.71	1.61	0.01	59.71	10.01	70.41	2.21	26A
16.11	3.31	13.51	23.01	0.01	30.71	4.71	52.21	27.11	542
15.61	0.61	12.11	0.01	0.01	60.21	11.51	72.31	0.61	350
19.51	0.01	1.41	3.41	0.01	66.61	9.11	60.01	3.41	42A
10.91	3.51	12.71	25.01	0.01	35.01	5.01	47.61	20.51	40A
25.21	3.31	9.31	21.01	0.01	30.31	10.11	39.61	25.11	550C
17.61	11.11	13.21	14.11	1.31	20.71	14.01	43.21	25.21	59A
19.91	0.01	5.51	1.91	0.01	66.01	6.01	72.31	1.91	50C9
15.41	3.11	13.21	26.11	0.01	35.01	6.51	49.11	29.11	210
10.91	0.71	11.61	0.01	0.01	59.11	9.71	70.71	0.71	20
14.11	1.11	12.41	2.01	0.01	65.21	5.21	77.61	3.11	10
22.21	16.21	6.31	17.71	0.01	10.11	19.51	24.41	33.01	550
16.01	1.01	4.51	0.01	0.01	69.51	9.01	74.01	1.01	3A
17.01	4.31	12.41	29.01	0.01	34.01	2.51	46.41	34.11	90
15.41	4.31	0.71	13.21	0.01	53.21	5.11	62.01	17.51	024
14.21	3.11	12.01	20.01	1.11	35.21	14.31	40.31	23.21	020
16.01	14.61	10.11	16.01	0.61	27.71	14.01	30.51	30.71	200-1
23.21	1.91	0.31	1.11	2.31	22.71	40.41	33.31	3.01	440
24.61	15.01	0.71	16.01	0.01	15.31	19.61	24.01	31.01	540
15.51	0.01	1.31	2.91	0.01	72.71	7.61	74.01	2.91	55A
10.41	1.01	5.01	3.01	0.01	66.21	6.41	71.31	3.91	49A
10.61	15.31	9.41	10.01	0.01	27.31	11.41	36.71	33.31	51A
23.51	13.11	7.31	17.91	0.01	26.71	11.41	34.01	31.01	14A-1
13.31	1.11	14.51	0.91	0.01	64.61	5.61	79.11	2.01	250
22.91	2.41	10.31	21.51	0.01	26.51	16.41	36.91	23.91	41A
16.01	1.01	9.71	2.71	0.01	50.31	11.51	60.01	3.71	24A
0.41	0.51	14.11	10.21	2.11	27.61	37.01	43.01	10.01	61A

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.51	0.01	7.11	0.01	0.01	0.61	61.81	7.71	0.01	NRNG
10.21	14.91	0.31	10.71	0.01	24.41	15.51	32.71	33.61	17A
17.41	2.01	9.31	2.01	0.01	55.71	12.71	65.01	4.91	60A
14.11	0.01	13.01	1.21	0.01	67.01	3.11	80.01	2.01	26B
17.01	5.01	6.91	15.01	1.51	47.41	6.41	55.01	20.01	ATCC20800
16.01	0.01	1.01	2.01	0.01	70.01	9.41	72.61	2.01	2A
24.61	0.01	4.01	0.01	0.01	49.41	22.01	53.41	0.01	44A
17.41	1.01	0.01	2.91	0.01	55.31	23.31	55.31	4.61	14A
23.31	1.31	4.61	0.01	0.01	12.61	50.41	17.31	1.31	41B
19.31	0.01	1.11	3.01	0.01	66.61	9.11	67.01	3.01	66A
10.61	15.61	0.31	17.11	1.11	24.61	14.01	33.91	32.71	11A
19.61	5.11	10.11	27.21	0.01	77.51	10.61	37.51	32.31	21
15.71	2.41	14.01	25.71	0.01	36.71	5.41	50.01	20.11	33A
14.61	1.51	13.51	0.01	0.01	66.01	4.31	79.51	1.51	ATCC20822

PRIOR STRAINS

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
15.71	3.91	3.71	0.11	0.01	55.11	13.51	50.01	12.01	ATCC34304
20.21	1.61	6.91	11.41	0.01	17.01	34.11	24.71	12.91	ATCC24473
15.21	2.91	7.71	9.01	0.61	54.61	9.21	62.91	12.71	ATCC20211
23.21	10.71	4.31	12.61	1.51	20.61	27.01	26.41	23.41	ATCC20209
13.21	6.31	6.91	4.31	0.01	60.11	9.11	67.01	10.61	ATCC20210

TABLE 3: COMPOSITION OF OMEGA 3 FATTY ACID FRACTION

EPA	DPA	DHA	Strain
C20:5w3	C22:5w3	C22:6w3	
44.01	1.11	54.91	21
4.61	0.91	94.51	AICC20009
19.31	0.71	80.01	U40-2
31.91	0.01	60.11	210
07.91	0.01	12.11	BRBG1
12.51	6.11	81.51	56A
17.01	3.71	79.31	11A-1
24.91	4.31	70.81	4A-1
24.41	8.41	67.21	170
12.21	1.51	86.31	AICC20091
25.11	1.71	73.21	544
25.21	1.11	73.71	U30
16.21	5.41	78.41	59A
11.51	1.41	87.11	U37-2
14.01	1.91	84.21	550W
12.71	1.31	86.01	AICC20091
---	---	---	UX
21.01	2.91	76.11	LXW9
13.41	1.01	85.61	C32-2
15.01	4.31	80.71	5A-1
27.41	5.41	67.21	BRBG1
17.01	1.91	81.11	U3
20.51	1.31	78.21	550
19.01	5.01	74.41	18A
20.11	0.71	79.21	320
27.01	0.01	72.21	560
24.11	9.11	66.91	5X2
30.31	6.91	62.81	530
25.31	2.51	72.21	549
19.91	3.81	76.31	53
5.01	0.01	95.01	3A-1
36.91	2.61	60.51	15A
19.31	0.01	80.71	9A-1
25.81	4.41	69.81	510
26.31	5.01	68.71	8A-1
21.61	6.71	71.71	13A-1
28.01	0.01	72.01	240-2
28.71	0.01	71.31	240-1
16.21	0.01	83.81	30
6.31	0.01	93.71	50G5
19.71	3.31	77.01	160
25.71	2.11	72.61	6A-1
17.11	0.01	82.91	330
30.51	3.61	65.91	840
15.61	1.21	83.11	20A

LPA	DPA	DMA	Station
C20:5W3	C22:5W3	C27:6W3	
26.0X	0.0X	73.2X	43B
5.2Z	0.0X	94.0X	1A-1
17.4X	1.2X	81.5Z	U41-2
5.4X	0.0X	94.6X	56B
13.9X	1.3X	84.8X	46A
3.5X	0.0X	96.5X	15A-1
5.8X	2.4X	91.8X	13A
22.3X	0.0X	77.7X	37B
25.4X	0.0X	74.6X	43B
27.7X	1.9X	70.3X	17B
14.7X	0.0X	85.3X	27A
29.2X	0.0X	70.8X	46B
28.0X	7.5X	64.5X	ATCC20090
0.9X	0.0X	99.1X	5A
27.3Z	0.0X	72.7X	28B-2
16.9X	0.0X	83.1X	27B
34.3X	3.4X	62.3X	49B
9.7X	0.0X	90.3X	18B
26.1X	1.9X	71.9X	549-2
29.9X	0.0X	70.1X	20B
30.1X	6.2X	63.7X	8B
15.6X	1.5X	82.9X	13B
15.2X	0.0X	84.8X	26A
25.9X	0.0X	74.1X	54Z
16.7X	0.0X	83.3X	35B
2.1X	0.0X	97.9X	42A
26.6X	0.0X	73.4X	40A
23.4X	0.0X	76.6X	550C
30.6X	2.9X	66.4X	59A
7.6X	0.0X	92.4X	58G9
27.0X	0.0X	73.0X	21B
16.4X	0.0X	83.6X	2B
15.9X	0.0X	84.1X	1B
25.9X	0.0X	74.1X	55B
6.0X	0.0X	94.0X	3A
26.7X	0.0X	73.3X	9B
14.1X	0.0X	85.9X	U24
24.9X	2.2X	72.9X	U2B
26.4X	1.5X	72.1X	28B-1
24.8X	6.9X	68.3X	44B
36.4X	0.0X	63.6X	54B
1.8X	0.0X	98.2X	55A
7.1X	0.0X	92.9X	49A
25.6X	0.0X	74.4X	51A
21.5X	0.0X	78.5X	14A-1
18.4X	0.0X	81.6X	25B
28.1X	0.0X	71.9X	41A
14.3X	0.0X	85.7X	24A
32.3X	4.8X	63.0X	61A
91.6X	0.0X	8.4X	888G

CPA	UPA	OPA	Strain
C20:5w3	C22:5w3	C22:6w3	
25.51	0.01	74.51	17A
14.41	0.01	85.61	60A
16.11	0.01	83.91	26B
12.41	2.71	84.91	ATCC20080
2.51	0.01	97.51	2A
7.51	0.01	92.51	44A
0.01	0.01	100.01	14A
26.71	0.01	73.31	41B
1.71	0.01	98.31	66A
24.51	3.11	72.41	11A
26.81	0.01	73.21	2X
27.61	0.01	72.41	33A
17.01	0.01	83.01	ATCC20092

כך

Fig. 1 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and less than 10.6% omega-6 fatty acids (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids (as % of total fatty acids) and greater than 10.6% omega-6 (as % of total fatty acids).

Fig. 2 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and greater than 7.5% C20:5n-3 (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids (as % of total fatty acids) and less than 7.8% C20:5n-3 (as % of total fatty acids).

Example 5. Enhanced Growth Rates of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Previously Known Strains)

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888), *Schizochytrium* sp. S8 (ATCC No. 20889), *Thraustochytrium* sp. S42, *Thraustochytrium* sp. U42-2, *Thraustochytrium* sp. S42 and U30, (all isolated by the method of Example 1) and *Thraustochytrium aureum* (ATCC #28211) and *Schizochytrium aggregatum* (ATCC #28209) (previously known strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was transferred to another flask of M-5 medium and placed

on the shaker for 2 days. The cultures (1-2ml) were then transferred to another flask of M-5 medium and placed on the shaker for 1 day. This process ensured that all cultures were in the exponential phase of growth. These later

5 cultures were then used to inoculate two 250ml flasks of M-5 medium for each strain. These flasks were then placed on shakers at 25°C and 30°C, and changes in their optical density were monitored on a Beckman DB-G spectrophotometer (660nm, 1cm path length). Optical density readings were

10 taken at the following times: 0, 6, 10, 14, 17.25, 20.25 and 22.75 hours. Exponential growth rates (doublings/day) were then calculated from the optical density data by the method of Sorokin (1973). The results are presented in Table 4 and illustrated (normalized to the growth of strain U30 at 25°C)

15 in Fig. 4. The data indicate that the strains isolated by the method in Example 1 have much higher growth rates than the previously known ATCC strains at both 25°C and 30°C, even under the optimized phosphate levels essential for continuous growth. Strains of Thraustochytriales isolated from cold

20 Antarctic waters have not been shown to grow at 30°C.

Table 4. Exponential Growth Rate (doublings/day)

Strain	25°C	30°C
5 S31* (ATCC No. 20888)	8.5	9.4
U40-2*	5.8	6.0
10 S8* (ATCC No. 20889)	7.1	8.8
S42*	6.6	8.3
U30*	5.5	7.3
15 28209**	4.6	5.0
28210**	3.5	4.5
20 28211**	4.2	5.7
34304**	2.7	3.7
25 24473**	4.6	5.3

* strain isolated by method in Example 1

30 ** previously known ATCC strain

35 Example 6. Enhanced Production Characteristics (Growth and Lipid Induction) of Strains Isolated by Method in Example 1 Compared to ATCC Strains (Prior Art Strains)

Cells of *Schizochytrium* sp. S31 (ATCC No. 20888), *Schizochytrium* sp. S8 (ATCC No. 20889) (both isolated by the method of Example 1) and *Thraustochytrium aureum* (ATCC #28211) and *Schizochytrium aggregatum* (ATCC #28209) (prior art strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium (see Example 3). The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was transferred to another flask of M-5 medium and placed on the shaker for 2

days. The ash-free dry weights for each of these cultures were then quickly determined and then 3.29mg of each culture was pipetted into two 250ml erlenmeyer flasks containing 50ml of M-5 medium. These flasks were placed on a rotary shaker (200 rpm, 27°C). After 24 hours 20ml portions of each culture were then centrifuged, the supernatants discarded, and the cells transferred to 250ml erlenmeyer flasks containing 50 ml of M-5 medium without any glutamate (N-source). The flasks were placed back on the shaker, and after another 12 hours they were sampled to determine ash-free dry weights and quantify fatty acid contents by the method of Lepage and Roy (1984). The results are illustrated (normalized to the yields of ATCC No. 28211, previously known strain) in Fig. 5. The results indicate that the strains isolated by the method of Example 1 produced 2-3 times as much ash-free dry weight in the same period of time, under a combination of exponential growth and nitrogen limitation (for lipid induction) as the prior art ATCC strains. In addition, higher yields of total fatty acids and omega-3 fatty acids were obtained from strains of the present invention with strains S31 (ATCC No. 20888) producing 3-4 times as much omega-3 fatty acids as the prior art ATCC strains.

Example 7. Enhanced Lower Salinity Tolerance and Fatty Acid Production by Strains Isolated by Method in Example 1

Strains of 4 species of Thraustochytrids, *Schizochytrium* sp. S31 (ATCC No. 20888) and *Thraustochytrium* sp. U42-2 (ATCC No. 20891) (both isolated and screened by the method of

Example 1), and *S. aggregatum* (ATCC 28209) and *T. aureum* (ATCC 28210) (obtained from the American Type Culture Collection) were picked from solid F-1 medium and incubated for 3-4 days at 27°C on a rotary shaker (200 rpm). A range of differing salinity medium was prepared by making the following dilutions of M medium salts (NaCl, 25g/l; MgSO₄·7H₂O, 5g/l; KCl, 1g/l; CaCl₂, 200mg/l: 1) 100% (w/v M medium salts; 2) 80% (v/v) M medium, 20% (v/v) distilled water; 3) 60% (v/v) M medium, 40% (v/v) distilled water; 4) 40% (v/v) M medium, 60% (v/v) distilled water; 5) 20% (v/v) M medium, 80% distilled water; 6) 15% (v/v) M medium, 85% (v/v) distilled water; 7) 10% (v/v) M medium, 90% (v/v) distilled water; 8) 7% (v/v) M medium, 93% (v/v) distilled water; 9) 3% (v/v) M medium, 97% (v/v) distilled water; 10) 1.5% (v/v) M medium, 98.5% (v/v) distilled water. The following nutrients were added to the treatments (per liter): glucose, 5g; glutamate, 5g; yeast ext., 1g; (NH₄)₂SO₄, 200 mg; NaHCO₃, 200 mg; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotics solution, 2ml. Fifty ml of each of these treatments were inoculated with 1ml of the cells growing in the F-1 medium. These cultures were placed on an orbital shaker (200 rpm) and maintained at 27°C for 48 hr. The cells were harvested by centrifugation and total fatty acids determined by gas chromatography. The results are illustrated in Fig. 6. *Thraustochytrium* sp. U42-2 (ATCC No. 20891) isolated by the method of Example 1 can yield almost twice the amount of fatty acids produced by *T. aureum* (ATCC 28211) and over 8 times the amount of fatty acids

produced by *S. aggregatum* (ATCC 28209). Additionally, U42-2 appears to have a wider salinity tolerance at the upper end of the salinity range evaluated. *Schizochytrium* sp. S31 (ATCC No. 20888), also isolated by the method in Example 1, exhibited both a high fatty acid yield (2.5 to 10 times that of the previously known ATCC strains) and a much wider range of salinity tolerance than the ATCC strains. Additionally, *Schizochytrium* sp. S31 (ATCC No. 20888) grows best at very low salinities. This property provides a strong economic advantage when considering commercial production, both because of the corrosive effects of saline waters on metal reactors, and because of problems associated with the disposal of saline waters.

Example 8. Cultivation/Low Salinity

Fifty ml of M/10-5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) picked from an agar slant. The M/10-5 media contains: 1000ml deionized water, 2.5g NaCl, 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g KCl, 0.02g CaCl_2 , 1.0g KH_2PO_4 , 1.0g yeast extract, 5.0g glucose, 5.0g glutamic acids, 0.2g NaHCO_3 , 5ml PII trace metals, 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate a 2 liter fermenter containing 1700ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M/10-40 media). The

fermenter was maintained at 30°C, with aeration at 1
vol/vol/min, and mixing at 300 rpm. After 48 hr, the
concentration of cells in the fermenter was 21.7g/l. The
cells were harvested by centrifugation, lyophilized, and
5 stored under N₂.

The total fatty acid content and omega-3 fatty acid
content was determined by gas chromatography. The total
fatty acid content of the final product was 39.0% ash-free
dry weight. The omega-3 HUFA content (C20:5n-3, C22:5n-3
10 and C22:6n-3) of the microbial product was 25.6% of the total
fatty acid content. The ash content of the sample was 7.0%.

Example 9. Diversity of Fatty Acid Content

Growth and gas chromatographic analysis of fatty acid
15 production by various strains as described in Example 4
revealed differences in fatty acid diversity. Strains of
the present invention synthesized fewer different fatty acids
than previously available strains. Lower diversity of fatty
acids is advantageous in fatty acid purification since there
20 are fewer impurities to be separated. For food supplement
purposes, fewer different fatty acids is advantageous because
the likelihood of ingesting unwanted fatty acids is reduced.
Table 5 shows the number of different HUFAs present, at
concentrations greater than 1% by weight of total fatty acids
25 for previously known strains, designated by ATCC number and
various strains of the present invention.

Table 5.

5	Strain	No. of Different Fatty Acids at 1% or Greater % of Total Fatty Acids	
	34304**	8	
10	28211**	8	
	24473**	10	
	28209**	13	
15	28210**	8	
	S31*	5	
20	S8*	6	
	79B*	6	

25 * strain isolated by the method in Example 1

** previously known ATCC strain

30 Example 10. Recovery

Fifty ml of M5 culture media in a 250 ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) picked from an agar slant. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate a 1 liter fermenter containing 1000ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M20 media). The fermenter was maintained at 30°C and pH 7.4, with aeration at 1 vol/min, and mixing at 400 rpm. After 48 hr, the concentration of the cells in the fermenter was 18.5g/l. Aeration and mixing in the fermenter was turned off. Within 2-4 minutes, the

cells flocculated and settled in the bottom 250 ml of the fermenter. This concentrated zone of cells had a cell concentration of 72g/l. This zone of cells can be siphoned from the fermenter, and: (1) transferred to another reactor for a period of nitrogen limitation (e.g., combining the highly concentrated production of several fermenters); or (2) harvested directly by centrifugation or filtration. By preconcentrating the cells in this manner, 60-80% less water has to be processed to recover the cells.

10

Example 11. Utilization of a Variety of Carbon and Nitrogen Sources

Fifty ml of M5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of *Schizochytrium* sp. S31 (ATCC No. 20888) or *Thraustochytrium* sp. U42-2 (ATCC No. 20891) picked from an agar slant. The M5 media was described in Example 3 except for the addition of 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. This culture was used to inoculate flasks of M5 media with one of the following substituted for the glucose (at 5g/l): dextrin, sorbitol, fructose, lactose, maltose, sucrose, corn starch, wheat starch, potato starch, ground corn; or one of the following substituted for the glutamate (at 5g/l): gelysate, peptone, tryptone, casein, corn steep liquor, urea, nitrate, ammonium, whey, or corn gluten meal. The cultures were incubated for 48 hours on a rotary shaker (200 rpm, 27°C).

The relative culture densities, representing growth on the different organic substrates, are illustrated in Tables 6-7.

Table 6. Utilization of Nitrogen Sources

5	N-Source	Strains	
		<i>Thraustochytrium</i> sp. U42-2 ATCC No. 20891	<i>Schizochytrium</i> sp. S31 ATCC No. 20888
10			
	glutamate	+++	+++
15	gelysate	+++	+++
	peptone	++	++
	tryptone	++	++
20	casein	++	++
	corn steep liquor	+++	+++
25	urea	+	++
	nitrate	++	+++
30	ammonium	+	+++
	whey	+++	+++
35	corn gluten meal	+++	+++
	+++ = high growth		
	++ = medium growth		
40	+ = low growth		
	0 = no growth		

Table 7. Utilization of Organic Carbon Sources

C-Source		Strains	
		<i>Thraustochytrium</i> sp. U42-2 ATCC No. 20891	<i>Schizochytrium</i> sp. S31 ATCC No. 20888
5			
10	glucose	+++	+++
	dextrin	+++	+++
	sorbitol	+	+
15	fructose	+	+++
	lactose	+	+
20	maltose	+++	+
	sucrose	+	+
	corn starch	+++	+
25	wheat starch	+++	+
	potato starch	+++	+
30	ground corn	+++	0
	+++ = high growth		
	++ = medium growth		
35	+ = low growth		
	0 = no growth		

40 Example 12. Feeding of *Thraustochytrid*-Based Feed Supplement to Brine Shrimp to Increase Their Omega-3 HUFA Content

Cellular biomass of *Thraustochytrium* sp. 12B (ATCC 20890) was produced in shake flasks in M-5 medium (see Example 3) at 25°C. Cellular biomass of *Thraustochytrium* sp. S31 (ATCC 20888) was produced in shake flasks in M/10-5 medium (see Example 8) at 27°C. The cells of each strain were harvested by centrifugation. The pellet was washed once with distilled water and recentrifuged to produce a 50% solids paste. The resulting paste was resuspended in sea

water and then added to an adult brine shrimp culture as a feed supplement. The brine shrimp had previously been reared on agricultural waste products and as a result their omega-3 HUFA content was very low, only 1.3 - 2.3% of total fatty acids (wild-caught brine shrimp have an average omega-3 HUFA content of 6 - 8% total fatty acids). The brine shrimp (2 - 3/mL) were held in a 1 liter beaker filled with sea water and an airstone was utilized to aerate and mix the culture. After addition of the feed supplement, samples of the brine shrimp were periodically harvested, washed, and their fatty acid content determined by gas chromatography. The results are illustrated in Figs. 7 and 8. When fed the thraustochytrid-based feed supplement as a finishing feed, the omega-3 content of the brine shrimp can be raised to that of wild-type brine shrimp within 5 hours if fed strain 12B or within 11 hours when fed strain S31. The omega-3 HUFA content of the brine shrimp can be greatly enhanced over that of the wild type if fed these feed supplements for up to 24 hours. Additionally, these feed supplements greatly increase the DHA content of the brine shrimp, which is generally only reported in trace levels in wild-caught brine shrimp.

Example 13. Use of Sodium Sulfate in Culture Medium

This example illustrates that omega-3 production and total fatty acid content is not harmed and can be the same or better when using sodium sulfate instead of sodium chloride as the sodium salt in a fermentation medium.

Schizochytrium ATCC No. 20888 was grown in medium, pH 7.0, containing 2.36 grams of sodium per liter of medium, 1.5-3.0 grams of a nitrogen source per liter of medium, and 3.0 grams of glucose per liter of medium. The cells were incubated at 28°C, at 200 rotations per minute, for 48 hours. The results are shown in Table 8.

Table 8. Effect of Sodium Sulfate Compared With Sodium Chloride on Fatty Acid Content

10	A) Na salt = <u>sodium chloride</u> ; N source = sodium glutamate			
15	N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
	3.0	6.0	11.2	1.74
	2.5	5.8	10.8	1.71
20	2.0	5.8	11.0	1.65
	1.5	7.5	20.3	1.39
25	B) Na salt = <u>sodium chloride</u> ; N source = peptone			
	N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
30	3.0	7.9	21.9	1.34
	2.5	9.4	27.4	1.21
	2.0	6.7	28.9	1.18
35	1.5	11.1	42.1	1.16
40	C) Na salt = <u>sodium sulfate</u> ; N source = sodium glutamate			
	N source (g/L)	omega-3 (% dwt)	total fatty acid (% dwt)	biomass yield (g/L)
45	3.0	9.3	31.9	1.34
	2.5	10.1	38.6	1.35
	2.0	10.1	41.4	1.30
	1.5	9.5	43.6	1.26

As seen in Table 8, omega-3 and total fatty acid production when using sodium sulfate is comparable to or better than when using sodium chloride as a sodium salt.

5 Example 14. Production of *Schizochytrium* in Low Salinity Culture Medium

This Example illustrates the fermentation of *Schizochytrium* in a low salinity culture medium while maintaining high biomass yields and high omega-3 and fatty acid production.

Schizochytrium ATCC No. 20888 was grown in medium, containing 3.33g/l of peptone as a nitrogen source, 5.0g/l of glucose as a carbon source, with varying sodium concentrations. The cells were fermented at 30°C with an inoculum of about 40mg/L dwt for a period of 48 hours. The sodium was supplied as sodium chloride. The results of this run are shown in Table 9.

20 Table 9. Production of *Schizochytrium* in Low Salinity Culture Medium

25

Na conc. g/L	Cl conc. g/L	Biomass Yield g/L	Fatty acids % dwt	omega-3 % dwt	final glucose g/L
4.88	7.12	1.76±0.60	35.4±1.0	10.2±0.6	0.00
3.90	5.70	1.72±0.67	37.0±0.7	11.1±0.3	0.15
2.93	4.27	1.70±0.42	43.0±0.2	12.1±0.1	0.22
1.95	2.85	1.66±0.57	29.8±0.7	9.3±0.1	1.55
0.98	1.42	0.40±0.61	10.6±2.4	4.0±1.0	4.31

1047030
As can be seen from the results in Table 9, high biomass yields and production of omega-3 fatty acids and total fatty acids can be achieved at sodium concentrations of greater than about 1.0 g/l.

Example 15. Cultivation of *Schizochytrium* in Medium with Low Chloride Content

This Example illustrates the fermentation of microflora of the present invention at minimal chloride concentrations while achieving high biomass yields based on starting sugar concentration.

Schizochytrium ATCC No. 20888 was cultured in shake flasks at 200 rpm and 28°C in 50ml aliquots of the following medium. 1000ml deionized water; 1.2g Mg SO₄·7H₂O; 0.067g CaCO₃; 3.0g glucose; 3.0g monosodium glutamate; 0.2g KH₂PO₄; 0.4g yeast extract; 5.0ml PII metals, 1.0 vitamin mix; and 0.1g each of penicillin-G and streptomycin sulfate. The chloride concentration was varied by adding differing amounts of KCl to each treatment. The potassium concentration in all of the treatments was held constant by additions of potassium citrate. Sodium concentration was either 2.37g/l or 4.0 g/l through addition of sodium sulfate. The results of these fermentations are shown below in Table 10.

Table 10. Fermentation of *Schizochytrium* at Minimal Chloride Concentrations

Chloride conc. (mg/L)	Na 2.37 g/L Biomass Yield (mg/L)	Na 4.0 g/L Biomass Yield (mg/L)
0.1	198 ± 21	158 ± 48
7.1	545 ± 120	394 ± 151
15.1	975 ± 21	758 ± 163
30.1	1140 ± 99	930 ± 64
59.1	1713 ± 18	1650 ± 14
119.1	1863 ± 53	1663 ± 46
238.1	1913 ± 11	1643 ± 39

As can be seen from the results shown in Table 10, high yields of biomass per sugar can be achieved at low chloride concentrations. For example, at a chloride concentration of greater than 59.1mg/L, yields of greater than 50% are achieved.

Example 16. Variation of Sodium Sulfate Concentration at Low Chloride Concentrations

This Example illustrates the effect of varying sodium sulfate concentration in a fermentation at low chloride concentration.

Schizochytrium ATC 20888 was cultured in shake flasks at 200 rpm and 28°C in 50 ml aliquots of the following medium: 1000ml deionized water; 1.2g $MgSO_4 \cdot 7H_2O$; 0.125g KCl; 0.067g $CaCO_3$; 3.0g glucose; 3.0g monosodium glutamate; 0.2g KH_2PO_4 ; 0.4g yeast extract; 5.0ml PII metals; 1.0ml vitamin mix; and 0.1g each of penicillin-G and streptomycin sulfate. The sodium sulfate concentration was varied in the treatments from 3.0g/l to 30.2g/l. The results of the fermentation runs are shown below in Table 11.

Table 11. Variation of Sodium Sulfate Concentration at Low Chloride Content

	Sodium Sulfate (g/l)	Biomass yield (g/l)
25	3.0	0.78
30	6.0	1.13
	9.1	1.72
	12.1	1.88
	15.1	1.89
	22.7	1.91
35	30.2	1.63

The results shown in Table 11, illustrate that at a low chloride concentration of about 59g/l, high biomass yields from glucose of greater than 50% can be obtained by selection of an appropriate sodium sulfate concentration.

5

00461209-12490